# A beginner's guide to lipidomics

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Lipidomics refers to the large-scale analysis of the complete set of lipids – the 'lipidome' – in any biological system. Methodologically, it heavily relies on mass spectrometry, an analytic technique enabling the identification and quantification of molecules in a complex sample based on slight differences in their mass and charge. Recent advances in this field have fuelled the development of novel approaches including tracer lipidomics and spatial lipidomics, allowing an unprecedented insight into this complex class of biomolecules. As lipids play numerous physiological roles and are affected in a wide range of pathologies, the study of lipids and their metabolic pathways offers great potential for biomarker discovery and for the development of novel therapeutic interventions.

Lipids form a complex class of biomolecules that are insoluble in water. To date, close to 50,000 different lipid species have been identified, with many more theoretically possible. Major lipid classes include fatty acids, phospholipids, triglycerides, sterols and waxes. They all have different chemical structures that determine their biophysical properties and biological functions. Lipids are used as building blocks for the formation of cellular membranes; they play a role in energy storage and act as important signalling molecules. They contribute to numerous diseases including diabetes, obesity, liver disease, cancer and neurodegeneration and are of interest in any biological or biotechnological discipline ranging from agriculture and food industry to drug delivery. Because of their diversity and insolubility in water, lipids have been more difficult to study than other biomolecules such as DNA and proteins, which consist of a repetition of a small number of easily distinguishable building blocks, allowing them to be easily 'sequenced'. Historically, chromatographic methods such as thin layer chromatography (TLC) and gas chromatography (GC) have been used to study lipids, but these only allow to distinguish lipid classes or lack the resolving power to study unique complex lipid species. It is only with the progress in mass spectrometry (MS) that a more comprehensive analysis of the lipidome has become possible.

## Lipidomics

Lipidomics usually refers to the analysis of lipids in bulk in an organic extract of a biological sample. This can be any type of biological sample, including tissue, body fluids, plants or processed food. Most commonly, chloroform-containing liquid–liquid extraction methods, such as the Bligh–Dyer and Folch extraction protocols, are used. In these procedures, the sample is homogenized (in case of tissue) and a mixture of chloroform and methanol is added as well as an antioxidant to prevent oxidation of double bonds and a lipid standard mix for later correction of eventual differences in lipid extraction efficiency and ionization (as detailed later; Figure 1). Lipids are recovered in the organic phase after centrifugation and evaporation. Alternatively, the raw extract is applied to a resin in a column for solid phase extraction and elution. Lipid extracts are then subjected to MS. This can be done directly without any up-front separation, referred to as 'shotgun lipidomics'. Alternatively, lipids are separated by liquid chromatography (LC) before MS analysis. This set-up is referred to as LC-MS. LC separation of lipids can be based on the hydrophobicity of the fatty acyl chains (reversed phase chromatography, e.g., using a C18 column) or based on the hydrophilicity of the headgroups (HILIC).

A prerequisite for the analysis of lipids (and any other type of molecule) by MS is that they need to have a charge. In standard lipidomics, this is achieved by electrospray ionization (ESI; Figure 2). This is an ionization technique that involves the generation of a spray of small droplets through a capillary on which a high voltage is placed. After evaporation the resulting lipid ions enter the vacuum chamber of the mass spectrometer which contains mass analysers that separate ions based on small differences in their mass relative to their charge ($m/z$). In a classical set-up, quadrupole analysers are used as mass filters. A quadrupole consists of four parallel cylindrical metal rods on which a radiofrequency voltage is applied. Depending on the voltage, ions with a specific mass-to-charge ratio travel through the quadrupole, while other ions collide with the rods. By continuously varying the applied voltage, ions with different $m/z$ values are selected. This way, intact lipid species are separated from each other based on slight differences in their masses. To better identify and distinguish these lipids, ions that leave the quadrupole are fragmented by collision with an inert gas in a so-called collision cell. Depending on the collision energy, lipids can be fragmented at different positions, e.g., after the headgroup in the case of phospholipids. The resulting fragments are then separated in a second mass analyser before reaching the detector. This procedure is referred to as MS/MS. Based on the masses and intensities of the detected fragments, lipids can be identified and quantitated. This is done with the aid of databases and specific software



 

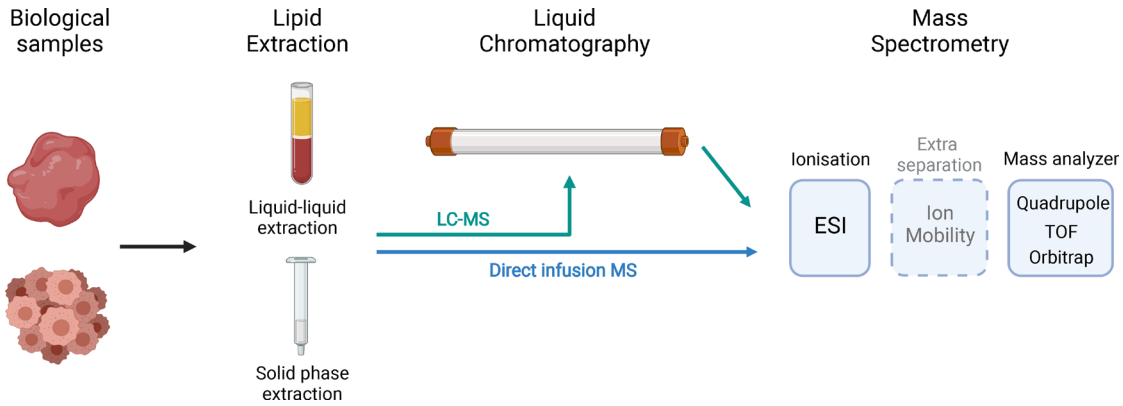


**Figure 1.** Overview of a mass spectrometry-based bulk lipidomics set-up. Lipids are extracted from homogenized biological samples by either liquid–liquid extraction or solid phase extraction. Crude lipid extracts can be directly injected into the mass spectrometer ('shotgun lipidomics') or can be separated by LC first. ESI is the preferred mode of ionization for the vast majority of lipids, while the choice of mass analyser often depends on whether the experiment is targeted or untargeted (discussed in the text). (This image was created with BioRender.com.)

tools. Most of these come with the instrument, although a few platform-independent data analysis packages have become available.

### *Challenges and solutions*

One of the challenges related to MS-based lipidomics is the complexity of the sample with many diverse species in

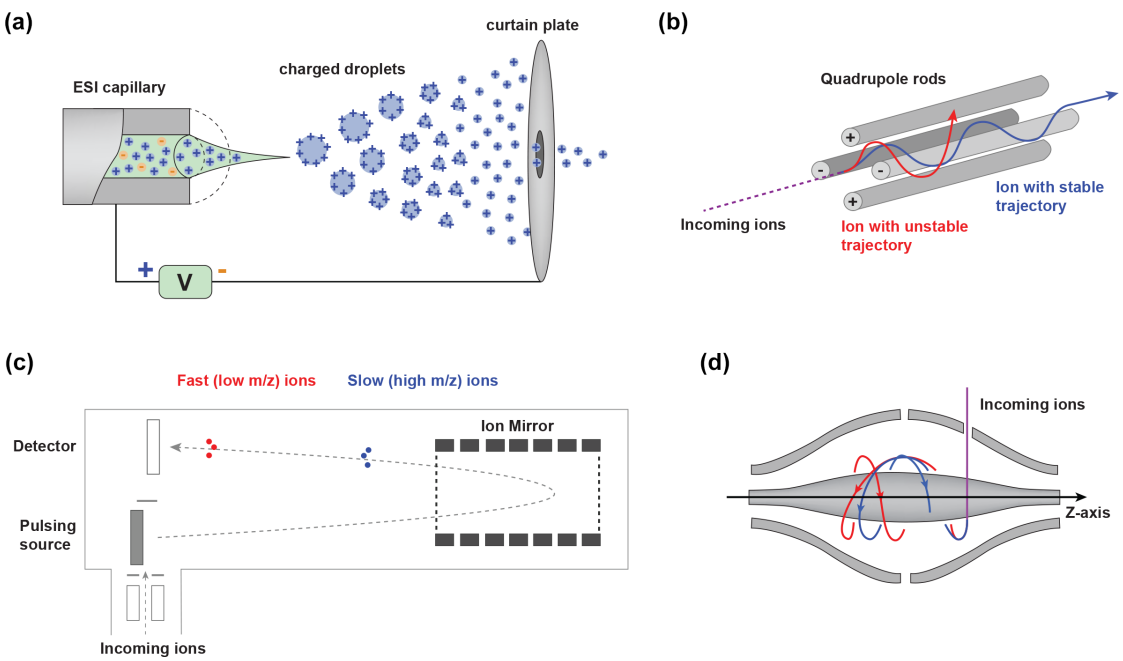


**Figure 2.** Principles of ionization and mass spectrometry analysis commonly applied in lipidomics**.** (a) In electrospray ionization, a high voltage is applied to a capillary from which the sample is sprayed, resulting in a fine mist of charged droplets. As these droplets gradually evaporate the concentrating charge forces the analytes into an ionized form, which enters the MS through a small opening in the curtain plate. (b) A quadrupole acts as a mass filter by oscillating electric fields between the rods in such a way that only ions of a particular *m/z* have a stable trajectory. (c) In a time-of-flight analyser, ions are accelerated in successive pulses through a flight tube and reflected back by an ion mirror towards the detector. The time it takes the ions to reach the detector depends on their *m/z* value. (d) In an Orbitrap analyser, ions orbit around a central spindle and oscillate back and forth along the *Z*-axis. The frequency of this oscillation, after a Fourier transformation, reveals the *m/z* values of the orbiting ions.

a small mass range between 300 and 900 Da. As classical quadrupoles have a limited mass resolution (ability to separate molecules with different masses), usually multiple lipid species with similar masses (referred to as isobaric species) are co-detected, hampering the exact assignment of a mass to a specific individual lipid species. This can in part be solved by using mass spectrometers with higher resolution mass analysers such as the time-of-flight (TOF) analyser, which separates ions based on their *m*/*z*-dependent acceleration in a flight tube or the Orbitrap in which ions oscillate around an inner rod (Figure 2). By applying a Fourier transformation to the measured ion oscillations, Orbitraps can resolve very small differences in mass. Alternatively, or additionally, lipids can be pre-separated by LC as in the LC-MS set-up to reduce the complexity of the sample before mass spectrometry. In high-end MS instruments, additional in-line ion separation techniques are used such as ion mobility separation (IMS) to separate ions based on differences in their shape and therefore mobility through a gas. Depending on the set-up of the instrument, lipids can be identified and annotated at different levels of identification or uniqueness. The lowest level of identification is the sum notation, for instance PC34:1 which refers to phosphatidylcholine lipids with 34 carbons and one unsaturation in both acyl chains together. It does not specify the length of the individual acyl chain, nor the position of the double bond and hence can represent multiple lipid species. The highest level of identification, for instance PC(16:0/18:1(9cis)) indicates the exact position of acyl chains and double bonds and corresponds to only one lipid species.

While direct infusion or the so-called shotgun lipidomics is a feasible strategy on high-resolution instruments, most researchers have favoured LC-MS, due to the increased specificity and sensitivity that LC-based approaches can offer. MS/MS brings further specificity and is the key to unlocking the more detailed levels of identification that consider the identities of individual acyl chains.

Another challenge in MS-based lipidomics is related to the efficiency of ionization which varies from species to species, making exact quantification difficult. This can in part be solved by correcting the intensities relative to those of a mix of internal lipid standards that have been spiked in the sample at known concentrations prior to extraction. The lipidomics standards initiative (LSI) defines three different levels of quantitation accuracy based on the use of internal standards. Most commonly, one internal standard for each lipid class of interest is used (level two type quantification as defined by the LSI). In level one quantification, multiple internal standards are used per lipid class, such that species-specific analytical response can be considered, while level three quantification is based on a standard from a different lipid class than the analyte of interest. To increase ionization efficiency, in some cases salts such as ammonium acetate are added.

***Targeted versus untargeted approaches***

TOF and Orbitrap analysers are ideal to produce a complete scan of all the (lipid) ions present in the sample. This is referred to as untargeted lipidomics. This method is well suited for discovery purposes to explore the lipid composition of a sample. As untargeted analyses tend to suffer from limited sensitivity and quantitative abilities, targeted approaches are often used instead. In a targeted approach, a list of lipid species of interest is predefined. Quadrupole instruments are naturally suited for a targeted analysis, as specific voltages can be applied on the mass filters in a sequential manner corresponding to only the lipids of interest. This can be few to even several thousand species. This approach is referred to as 'multiple reaction monitoring' and allows a longer time for data integration of each individual species, enhancing the quantitative aspect of the measurement.

***Mediator, oxidative and redox lipidomics***

Standard bulk lipidomics mainly analyses structural lipids such as phospholipids and sphingolipids, and storage lipids, such as triacylglycerides. Other lipids that play a role in cell signalling, including mediator lipids such as prostaglandins, leukotrienes, resolvins and maresins, or oxidized and oxygenated lipids are usually several orders of magnitude less abundant and require special protocols for their enrichment and analysis. As some of these lipids are (stereo)isomers with identical masses, dedicated separation techniques are required for their unambiguous identification and analysis.

## Spatial lipidomics

Bulk lipidomics measures the lipid composition in an extract of a biological sample, for instance a human tissue. The obtained data thus represents the average lipid profile of the tissue, not taking into account regional heterogeneity due to cell-type composition. To reveal this heterogeneity, spatial lipidomics is used. In a typical set-up, referred to as MALDI, a thin frozen section of a tissue is fixed to a slide and is covered by a thin layer of matrix (Figure 3). The slide is then scanned by a laser, releasing lipid ions that are separated based on the time they require to travel through a vacuum tube, which is dependent on their mass. This way the lipid composition of the tissue is determined pixel by pixel and visualized in 2D as a coloured image of the tissue. With the latest high-end instruments, thousands of lipids can be identified at a spatial resolution close to a single cell level (10 μm).



 

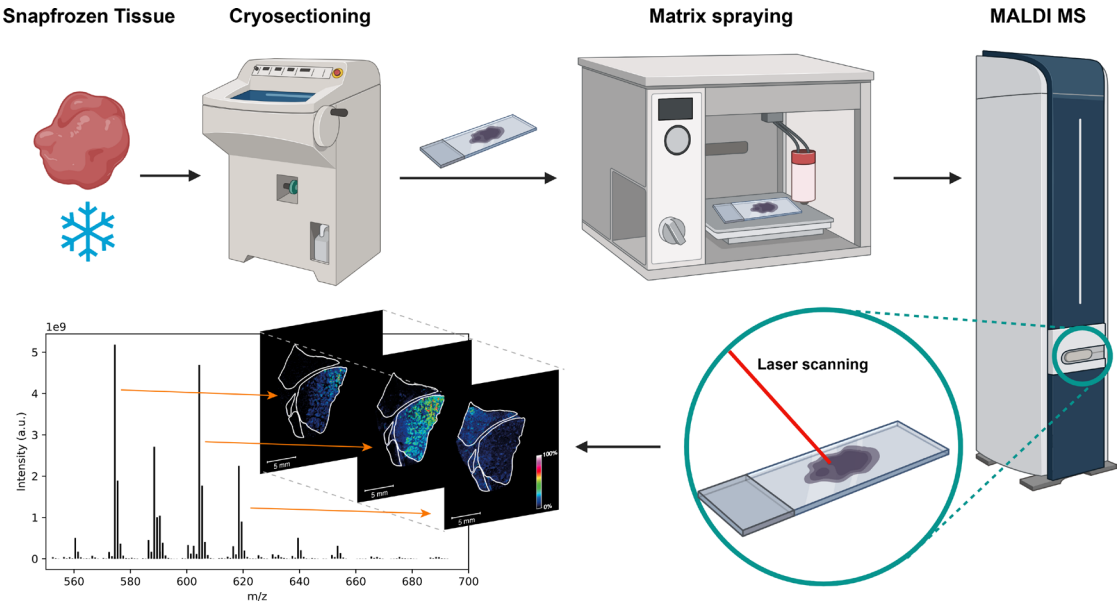


**Figure 3.** Overview of MALDI-based spatial lipidomics. (This image was created with BioRender.com.)

## Tracer lipidomics and pathway analysis

Classical bulk and spatial lipidomics measure steady-state levels of lipids in biological samples. This provides important information on eventual changes of lipids related to specific physiological or pathological conditions and finds major applications in biomarker development. It does not, however, provide direct insight into the complex pathways that underlie eventual changes in lipid profiles. An emerging approach to reveal changes in the fluxes of lipid pathways and in the link with the broader metabolic network is tracer lipidomics, also known as stable isotope-resolved lipidomics. In this approach, biological organisms are treated with stable isotopes (usually $^{13}$C-labelled substrates) such as fatty acids or glucose. Using high-resolution MS, the incorporation of $^{13}$C in various lipids is analysed, revealing dynamic changes in lipid metabolism pathways (Figure 4).

An alternative complementary approach to reveal changes in lipid metabolism involves the integration of lipidomics data with genomics, transcriptomics and proteomics. Recent advances in spatial multi-omics now allow us to place the lipidomics data in a systemic context with a spatial resolution down to the single cell level. This provides unique abilities to study the role of the lipidome in biological systems and provides unprecedented insight into its role in complex diseases.

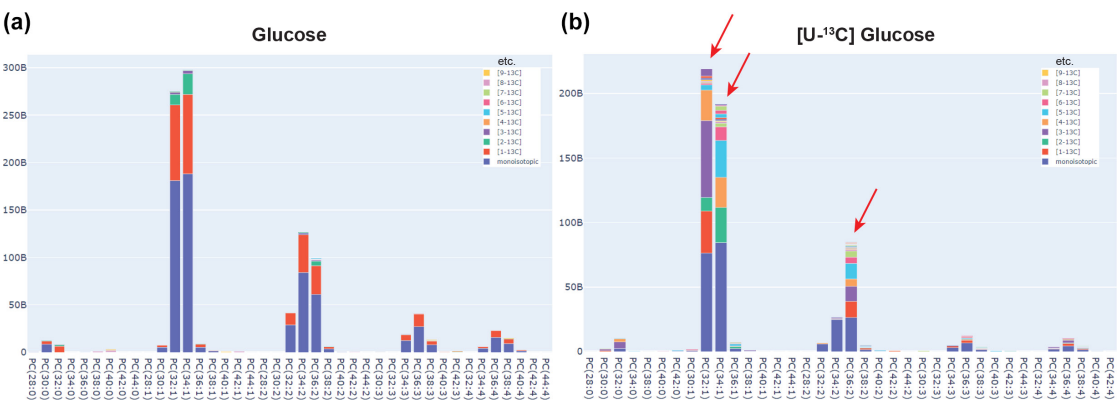


**Figure 4.** Pathway analysis by tracer lipidomics. (a) Phosphatidylcholine species of cells exposed to unlabelled glucose show monoisotopic species (blue) as well as *M*+1 and *M*+2 isotopologues due to the natural presence of $^{13}$C (red and green). (b) Exposure of cells to ubiquitously labelled glucose [U-$^{13}$C-glucose], reveals $^{13}$C incorporation in three mono-unsaturated species (PC32:1, 34:1 and 36:1) indicating the activity of glycolysis, the TCA cycle, *de novo* lipogenesis and lipid desaturation pathways.

## 'Real-world' applications and future challenges

Although MS-based lipidomics is a young emerging science that still requires further development and fine-tuning, it has a plethora of applications in the broad field of biological sciences. These include product development and quality control in the food industry, including quality control of dairy products and development of healthier meat products or meat substitutes. In biomedical sciences, applications range from the development of biomarkers for human disease to the identification of pathways for therapeutic targeting. An interesting tool based on lipidomics is the iKnife, a surgical knife that heats tissue during surgery and provides the surgeon with real-time information on the disease state of the tissue based on the analysis of lipids present in the smoke produced by the knife. Alternatively, collected surgical tissue or tissue biopsies are sectioned and subjected to spatial lipidomics to reveal tissue characteristics and heterogeneity to aid the clinician in making clinical prognoses and in therapy selection. The widespread clinical application of lipidomics, however, awaits standardization of techniques among different labs. Organizations such as LIPIDMAPS, the International Lipidomics Society and EpiLipidNet play a key facilitating role in these efforts. Other challenges include the expansion of the range of lipids that can be routinely analysed. In most current approaches, this is limited to some 2000 unique lipid species. Although this usually represents more than 90% of the lipid mass in a biological sample, many low abundant lipids, perhaps with important signalling roles, remain under the radar. Similarly, in view of the heterogeneity of lipid profiles among different cell types and particularly also among subcellular organelles, improvements in sensitivity and spatial resolution are essential. The fast pace of developments in the field of MS as well as other chemical imaging tools such as Raman microscopy hold great potential for the further maturation of the lipidomics field and its routine application in solving a wide range of real-world biological questions. ■

### Further reading


- LIPIDMAPS (https://www.lipidmaps.org/)
- International Lipidomics Society (https://lipidomicssociety.org/)
- EpiLipidNet (https://www.epilipid.net/)
- Lipidomics Standards Initiative (https://lipidomics-standards-initiative.org/)
- Holčapek, M., Liebisch, G. and Ekroos, K. (2018) Lipidomic analysis. *Anal. Chem.* **90**, 4249–4257. DOI: 10.1021/acs.analchem.7b05395
- Züllig, T. and Köfeler, H.C. (2021) High resolution mass spectrometry in lipidomics. *Mass Spectrom. Rev.* **40**, 162–176. DOI: 10.1002/mas.21627
- Griffiths, W. and Wang, Y. (Eds.) (2020) Lipidomics: current and emerging techniques. Royal Society of Chemistry, Cambridge.
- Lange, M., Ni, Z., Criscuolo, A. and Fedorova, M. (2019) Liquid chromatography techniques in lipidomics research. *Chromatographia* **82**, 77–100. DOI: 10.1007/s10337-018-3656-4


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*Dr Jonas Dehairs is the operational manager and technology expert at the KU Leuven Lipidomics Core Facility Lipometrix. After obtaining his PhD in biomedical sciences he held a post-doctoral position at the laboratory of Prof Swinnen. In 2019, he took on his current position at Lipometrix where he established state-of-the-art targeted and untargeted lipidomics methods. His current research focus is on the development and implementation of untargeted tracer lipidomics methods to unravel complex lipid metabolic pathway activities.*



 